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Unique type of plasmid maintenance function: Postsegregational killing of plasmid-free cells

(plasmid stability/*parB*$^+$ of plasmid R1/regulated killing/phage λ promoter P_R)

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ABSTRACT **The stability locus *parB*$^+$ of plasmid R1 has been found to specify a unique type of plasmid maintenance function. Two genes, *hok* (*host killing*) and *sok* (*suppressor of killing*), are required for the stabilizing activity. The *hok* gene encodes a highly toxic gene product, whose overexpression causes a rapid killing and a concomitant dramatic change in morphology of the host cell. The other gene, *sok*, was found to encode a product that counteracts the *hok* gene-mediated killing. The *parB*$^+$ region was inserted in a plasmid with a temperature-sensitive replication system. At nonpermissive temperature, the *parB*$^+$ plasmid was maintained in the population for a significantly longer period than the corresponding *parB*$^-$ plasmid. Coupled to this extended maintenance, a large fraction of the population was shown to be nonviable plasmid-free cells with the characteristic *hok*-induced change in morphology. Based on these findings, we propose that the *parB*$^+$ locus mediates plasmid stability by killing cells that have lost the *parB*$^+$ plasmid during the preceding cell division, thereby ensuring that a growing bacterial culture predominantly consists of plasmid-containing cells.**

The stable maintenance of bacterial plasmids has been studied very actively for more than a decade, and in addition to the accumulation of detailed knowledge concerning plasmid replication and partitioning, the investigations have also contributed to a better understanding of problems of general interest within prokaryotic molecular biology.

The problem of stable maintenance in the absence of selection pressure is one that low copy number plasmids face continuously, and without strict control systems operating at the level of replication and replicon distribution (partitioning), the risk of irreversible plasmid loss would be overwhelming.

The solutions offered by various plasmids seem to be thorough, and an excellent example of this is illustrated in the great amount of work published about maintenance of plasmid F. In an elegant physiological analysis Tsutsui and Matsubara (1) demonstrated the fine tuning of the replication rate, and Ogura and Hiraga (2, 3) showed that two different sets of functions operate to ensure proper segregation of the plasmid molecules. One of these, encoded by the *sopA, -B, -C* region, most likely constitutes the "true" partition apparatus, whereas the other, encoded by the so-called *ccdA, -B* genes, rescues the plasmid molecule that happens to be present as a single copy in a cell at the point of division. In a yet unknown fashion, such a cell is blocked with respect to division until replication of the plasmid has ensured that proper segregation of the daughter replicons to the two new cells can take place. Thus, plasmid F apparently has taken care of all foreseeable maintenance problems and consequently is inherited with an extreme stability. Also, in the case of other low copy number plasmids (pSC101, P1), stabilization functions ensuring stable maintenance have been described (4, 5).

A member of another low copy number group (IncFII), plasmid R1, has also been the focus of considerable interest and, in analogy with the findings by Ogura and Hiraga for plasmid F, plasmid R1 also encodes two different sets of stability functions (6, 7). It was therefore obvious for us to speculate that similar activities were connected to what, in case of R1, has been termed *parA*$^+$ and *parB*$^+$. One of these loci, *parB*$^+$, has been especially interesting to us, since the presence of this region in all unstably inherited plasmids so far investigated (no matter what replicon) causes a very significant stabilization. In the present communication we show that the mechanism behind the *parB*$^+$-mediated stabilization is not related to any hitherto-described plasmid maintenance function, and on basis of the mechanism suggested it becomes understandable why any unstable plasmid should be stabilized by *parB*$^+$.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* K-12 strain CSH50 [Δ (*lac pro*) *rpsL* (8)] was used as the host strain for various plasmids in the genetic experiments, whereas *E. coli* K-12 strain JC411 [*metB leu his argG lacY malA xyl mtl gal rpsL* (9)] was used in the physiological experiments. The plasmids used and constructed are listed in Table 1.

Growth Medium and Measurements. The growth medium was either LB medium (10) supplemented with 0.2% glucose and 1 μg of thiamine per ml or A + B minimal medium (11) supplemented with 0.2% glucose and 1% Casamino acids. Growth of bacterial cultures was measured either as optical density at 450 nm using a Zeiss PM Q2 spectrophotometer or as viable counts measured by plating appropriate dilutions on selective or nonselective media.

Recombinant DNA Techniques. Clear lysates were prepared by the method of Clewell and Helinski (12). Small-scale preparation of plasmid DNA was done by the method of Birnboim and Doly (13). Large-scale preparation of plasmid DNA was done by the method of Stougaard and Molin (14). Digestion with restriction endonucleases was done essentially as recommended by the manufacturers (Boehringer Mannheim or New England Biolabs). Nick-translation of purified plasmid DNA was done essentially as recommended by the manufacturer (Amersham) using [^{32}P]dGTP for radioactive labeling.

Microscropic Observations. "Ghost" cells were detected by using a phase-contrast microscope (Olympus) at the highest magnification (10 × 100).

Separation of Cells on Percoll Gradients. Percoll (Pharmacia) gradients were performed by spinning a 50% Percoll solution at 13,000 rpm for 30 min in a fixed-angle SS34 Sorvall rotor. Samples of 1–2 ml of culture were layered carefully on top of the gradient, and the cells were banded by spinning at 5000 rpm for

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Abbreviation: bp, base pair(s).

Table 1. Properties of plasmids used and constructed

Plasmid	Type of replicon	Relevant genotype*	Coordinates of *parB* fragment,† bp	Ref. or source
pOU82	mini-R1	*bla*$^+$ *lac*$^+$ *par*$^-$		7
pPR95	mini-R1	*bla*$^+$ *lac*$^+$ *parB*$^+$	−300 to +580	Unpublished data
pOU491	pSC101	*bla*$^+$ *tet*$^+$ *rep*(ts)		4
pPR122	pSC101	*bla*$^+$ *tet*$^+$ *rep*(ts) *parB*$^+$	−300 to +580	This study
pPR633	pBR322	*bla*$^+$ *parB*$^+$	0 to +580	Unpublished data
pKG633	pBR322	*bla*$^+$ *parB*$^+$ cI_{857} P_R	0 to +580	This study
pPR634	pBR322	*bla*$^+$ *parB*$^+$	+194 to +580	Unpublished data
pKG634	pBR322	*bla*$^+$ *parB*$^-$ cI_{857} P_R	+194 to +580	This study
pBR341	pBR322	*bla*$^+$ *parB*$^-$	+268 to +580	Unpublished data
pKG341	pBR322	*bla*$^+$ *parB*$^-$ cI_{857} P_R	+268 to +580	This study
pPR171	pBR322	*bla*$^+$ *parB*$^-$	0 to +288	Unpublished data
pKG171	pBR322	*bla*$^+$ *parB*$^-$ cI_{857} P_R	0 to +288	This study
pML31	mini-F	*aphA*$^+$		15
pF634	mini-F	*aphA*$^+$ *parB*$^-$ cI_{857} P_R	+194 to +580	This study

*cI_{857} P_R denotes the presence of a DNA fragment containing the cI_{857} temperature-sensitive allele of the λ repressor gene and the λP_R promoter reading into the *parB*$^+$ region of the respective plasmid.
†Coordinates as in Fig. 3. The reference point (0 bp) is the *Bam*HI site in pPR633 that contains the minimal *parB*$^+$ region on a 580-base-pair (bp) *Bam*HI–*Eco*RI restriction fragment.

10 min. The density of the turbid bands appearing was determined by using density marker beads (Pharmacia).

Construction of Plasmids. All plasmids constructed are listed in Table 1. pPR122: The *parB Eco*RI fragment (900 bp) of pPR95 (mini-R1 *parB*$^+$) was inserted into the *Eco*RI site of pOU491, resulting in pPR122. pKG633: The *Sal* I–*Bgl* II fragment of pOU82 containing the cI_{857} temperature-sensitive allele of the λ repressor gene and the λ P_R promoter was inserted into pPR633 in front of the *parB*$^+$ region such that the P_R promoter reads into the region from left to right (see Fig. 3). In an analogous way, the *Sal* I–*Bgl* II fragment of pOU82 was inserted into pPR634 and pPR341, which are deletion derivatives of pPR633 (unpublished data), resulting in pKG634 and pKG341, respectively. The coordinates of the *parB*$^+$-derived fragments of these plasmids are given in Table 1. pKG171: In pPR171, the *Sal* I–*Bgl* II fragment of pOU82 was inserted in the opposite orientation, resulting in pKG171. The positions and orientations of the inserted P_R promoters relative to the *hok* and *sok* genes are shown in Fig. 3. pF634: The *Eco*RI–*Sal* I fragment of pKG634 containing the right 390 bp of the *parB*$^+$ region and the λ *c*I857-P_R inducible promoter system was inserted into the unique *Sal* I site in the kanamycin-resistance (*aphA*$^+$) fragment of pML31 by blunt-end ligation (S1 nuclease was used to make the restricted DNA fragment blunt-ended).

RESULTS

Is the *parB*$^+$ Region a *ccd* Analogue? The possibility that the *parB*$^+$ locus expresses a function that interferes with host cell division was tested through construction and analysis of plasmid pPR122. This plasmid is a *rep*(ts) derivative of pSC101 (pOU491) carrying a 900-bp *Eco*RI fragment with the *parB*$^+$ region. Thus, when cells carrying this plasmid are transferred to 42°C, which is restrictive for replication of pPR122, effects on cell growth should be detectable. The growth pattern of such cells (JC411 harboring either pOU491 or pPR122) is shown in Fig. 1.

Fig. 1*A* shows growth curves after a temperature shift as OD_{450} or viable counts. As expected, pOU491 has no effect on either viable counts or OD_{450}, whereas pPR122 causes the increment in viable counts to cease after approximately three generations after the temperature shift. This cessation is transient, lasting for a period of three or four generations. A significant reduction in rate is also observed for the OD_{450} curve. Fig. 1*B* shows viable counts on a selective medium that measures the number of plasmid-carrying cells in the two cultures: after the shift to nonpermissive temperature, there is again an increment for the first three generations, after which colony counts stay constant. The initial period of increasing cell numbers must correspond to cell divisions without plasmid replication. The estimated copy number of pSC101 is eight per cell (4); therefore, the period of three generations during which viable counts continue to increase on selective medium can be taken to reflect the time it takes to "dilute" the plasmid copies to one per cell. In other words, the plateau value represents the number of plasmid molecules present in the population at the time of temperature shift.

Fig. 1*C* shows the fraction of plasmid-carrying cells in the two strains. Both plasmids are stably maintained at 30°C (no plasmid loss is observed at all). After the shift to 42°C, pOU491 is stably maintained for three or four generations, after which the fraction of plasmid-carrying cells declines. In the case of the *parB*$^+$ plasmid (pPR122), plasmid loss is only observed after seven or eight generations.

These observations could be interpreted superficially to argue for a transient inhibition of cell division of cells harboring only one copy of pPR122. However, as will become apparent below, several lines of evidence disfavor this hypothesis.

A clear prediction from a *ccd*-like activity is the formation of filamentous cells when plasmid replication is blocked (3). Therefore, samples from the temperature-shift experiment (Fig. 1) were analyzed in a light microscope and in a Coulter Counter. These experiments showed no *parB*$^+$-mediated increase in cell size (data not shown). It should be noted in Fig. 1*A* that growth measured as optical density is reduced in rate sooner than would be expected from inhibition of cell division. Therefore, cells from the temperature-shift experiment were reexamined in the phase-contrast microscope and it was observed that samples from the culture harboring the *parB*$^+$ plasmid contained cells with a highly unusual morphology. A photograph of these cells is presented in Fig. 2. As seen here, the cells are transparent centrally, whereas the poles contain highly condensed material. Because of this translucent and apparently nonviable appearance, these cells are referred to as "ghosts". The kinetics of appearance of ghost cells in the temperature-shift experiment is shown in Fig. 1*C*. Ghost cell formation begins when plasmid-free cells start to appear in the control culture [JC411 (pOU491)].

Based on these observations, we find it very unlikely that *parB*$^+$ exerts a coupling to cell division like the mechanism proposed for *ccd* of plasmid F (3).

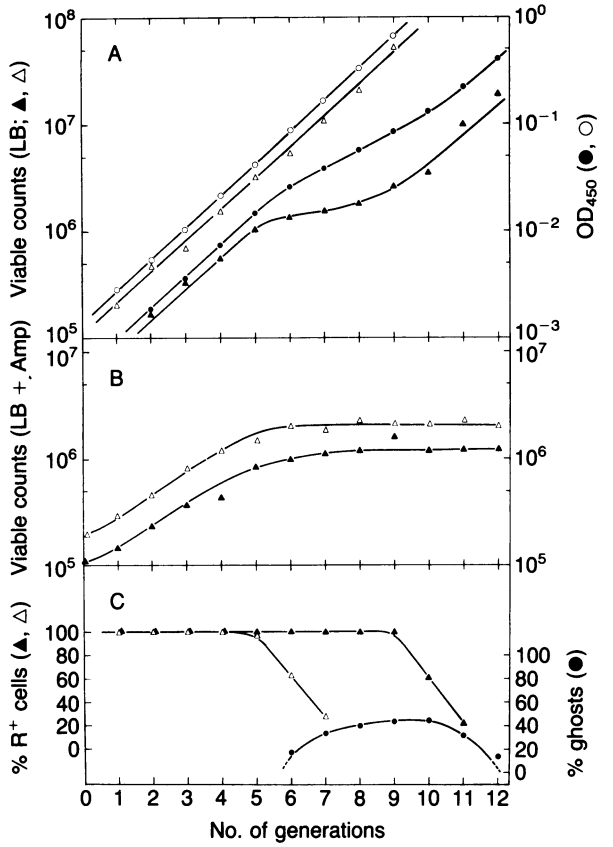

FIG. 1. Kinetics of cell growth, plasmid stability, and ghost cell formation after transfer to 42°C. Strain JC411 containing either pOU491 (open symbols) or pPR122 (closed symbols) was grown exponentially at 30°C in A + B minimal medium supplemented with 1% Casamino acids, and at time zero the cultures were shifted to 42°C. (*A*) Optical density at 450 nm and viable counts, which were determined as the numbers of colony formers at 30°C by plating appropriate dilutions on nonselective medium (LB plates). (*B*) Numbers of plasmid-carrying cells in the culture were determined as numbers of colony formers at 30°C on selective medium [LB plates containing either ampicillin (Amp) at 50 μg/ml or tetracycline at 10 μg/ml]. (*C*) Plasmid stability was measured by determining the fraction of plasmid-containing cells (% R^+ cells) by replica plating from nonselective medium (LB plates) to selective medium (LB plates containing ampicillin at 50 μg/ml); kinetics of ghost cell formation was determined by counting the fraction of cells with changed morphology using a phase-contrast microscope.

Characterization of *parB*$^+$-Induced Ghost Cells. Microscopic analysis (Fig. 2) of cultures developing ghost cells in the experiment described in Fig. 1 showed that at the maximum up to 35–40% of all cells had this changed morphology. Therefore, it should be possible to separate these from the rest and characterize them further. Samples of cells (1–2 ml of culture) were loaded on top of a Percoll gradient (0–100%) and centrifuged. In the culture containing pPR122 (*parB*$^+$) two turbid cell bands appeared, one banding at a density of 1.09 g/ml and a lighter band with a density of 1.06 g/ml. Only the heavier band was seen from the control culture. Microscope inspection showed that the lighter band consisted of ghost cells exclusively, whereas the other band contained normal cells and a few cells with changed morphology. The plating efficiency of the ghosts was extremely low compared to cells from the heavy band in the Percoll gradient. Thus, ghost cells are not viable.

Two observations indicate that ghost cells are plasmid-free. (*i*) In the temperature-shift experiment, viable counts on selective medium (30°C) stay constant (Fig. 1*B*) despite the presence of 35–40% dead cells in the culture (Fig. 1*C*). Thus, the cells that maintain one copy of the plasmid pPR122 seem to stay viable. (*ii*) DNA was prepared from cells harvested from the two bands of the Percoll gradient, nick-translated using DNA polymerase I (Klenow fragment), and separated on an agarose gel. Even after an extended exposure time (20 days), no radioactive plasmid band was visible in the DNA prepared from ghost cells, whereas such a band clearly showed up in the DNA preparation from the normal cells. These findings strongly suggest that the majority or all of the ghost cells are plasmid-free.

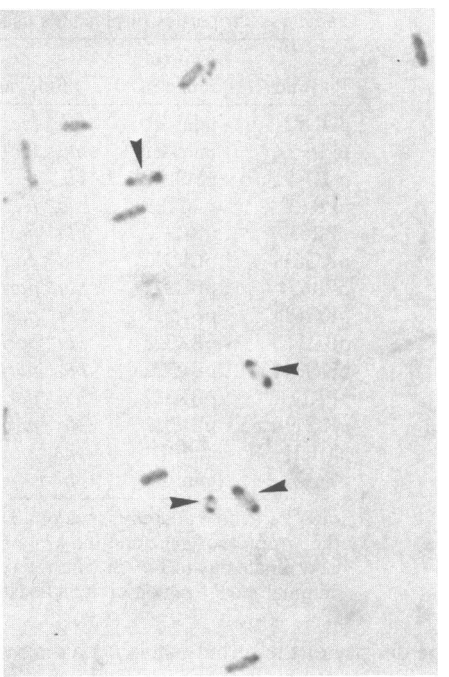

FIG. 2. Photograph of cells from the temperature-shift experiment (see Fig. 1) in a sample taken 9 or 10 generations after the temperature shift. Arrowheads point at cells with clearly changed morphology. Cells with a normal morphology are also seen. (×1600.)

The phenotype thus related to the *parB*$^+$ locus seems to be induction of a lethal function in cells that have lost a *parB*$^+$ plasmid during the preceding cell division. The gene associated with this phenotype is in the following termed *hok* (*h*ost *k*illing).

Transcriptional Induction of the *hok* Gene. Recently, the *parB*$^+$ region was mapped to a 1900-bp *Pst* I fragment located in one end of the largest *Eco*RI fragment of plasmid R1, as shown in Fig. 3 (7). By employing a BAL-31 deletion procedure, the *parB*$^+$ region was further mapped to lie within 580 bp, whose sequence was eventually determined (unpublished data).

As a first attempt to expose the nature and position of the *hok* gene, we made a series of insertions of a DNA fragment carrying the *c*I857 gene and the λ P_R promoter into pBR322 derivatives containing different parts of the right end of the *parB*$^+$ region (schematically shown in Fig. 3 as pKG633, pKG634, and pKG341). At 30°C cells harboring these plasmids grow normally; however, induction of P_R (at 42°C) results in rapid killing of the host cells and simultaneous appearance of ghost cells in the culture in all three cases.

Fig. 4 shows the killing kinetics (viable counts) and growth measured as OD_{450} after a shift to 42°C of strain JC411 (pKG634). Viable counts decrease rapidly (half-life, 2.5 min) and OD_{450} also drops significantly, indicating either actual cell lysis or a changed light scattering of the ghost cells. The presence of a λ P_R promoter transcribing the *parB*$^+$ region in the opposite direction (pKG171, Fig. 3) has no effect on cell growth and viability (Fig. 4, control). These results place the *hok* gene in the right-hand end of the *parB*$^+$ region (see Fig. 3) and also indicate that a gene product is the killing factor.

Suppression of the Host Killing Effect Expressed by *hok*. A gene from which a highly toxic product is expressed obvi-

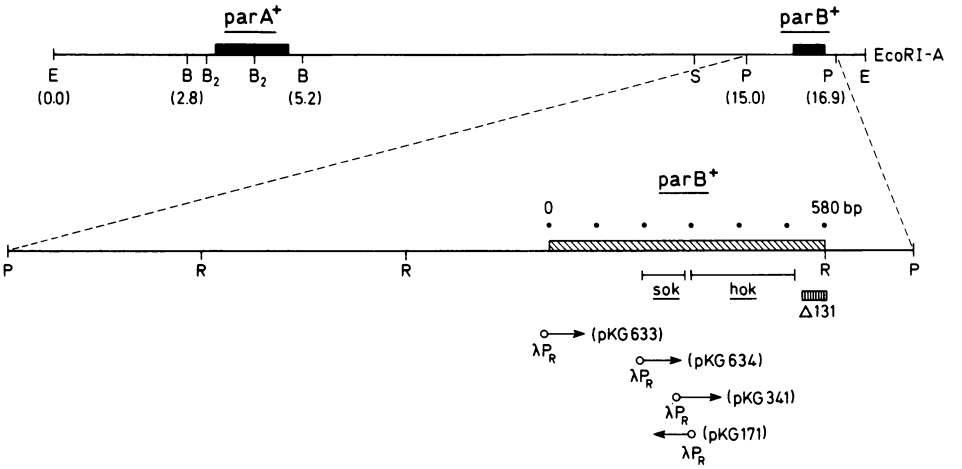

FIG. 3. Genetic map of the *par*$^+$ region from plasmid R1. The localizations of the *parA*$^+$ region and the *parB*$^+$ region within the *Eco*RI-A fragment of plasmid R1 are shown as black boxes. Restriction enzyme sites in the *Eco*RI-A fragment are essentially as described (7). The *parB*$^+$ region is located within the 1.9-kilobase *Pst* I fragment bordered by coordinates 15.0–16.9 (7). The *parB*$^+$ region was further mapped to the right-hand 580 bp of an 880-bp *Rsa* I fragment (unpublished data). The hatched region indicates the minimal *parB*$^+$ region. The positions of the *hok* and *sok* genes within the 580-bp *parB*$^+$ region are also shown. A *Bgl* II–*Sal* I fragment containing the P_R promoter and the cI_{857} allele of the λ repressor gene was inserted into pBR322 derivatives carrying various parts of the *parB*$^+$ fragment (unpublished data). The position of the inserted fragments and the direction of transcription from P_R are shown below the map of the *parB*$^+$ region (arrows). The λ P_R promoters in pKG633, pKG634, and pKG341 read from left to right into the *parB*$^+$ region, whereas the P_R promoter in pKG171 reads from right to left. Restriction enzyme sites are shown as E (*Eco*RI), B (*Bal* I), B_2 (*Bgl* II), S (*Sal* I), R (*Rsa* I), and P (*Pst* I).

ously must be regulated. Since the presence of *parB*$^+$ on a plasmid is harmless to the cells as long as there is no loss of the plasmid, we assumed that the regulator of *hok* was also encoded by the *parB*$^+$ region itself. In a first attempt to characterize this regulatory loop, the fragment of pKG634 containing λ cI_{857} and P_R upstream of the *hok* gene was inserted into a mini-F plasmid, resulting in pF634. Fig. 5 presents the induction kinetics of JC411 (pF634), which shows that the killing occurs somewhat slower and less efficiently than in the case of pKG634. Thus, the rate of killing is apparently gene dosage-dependent.

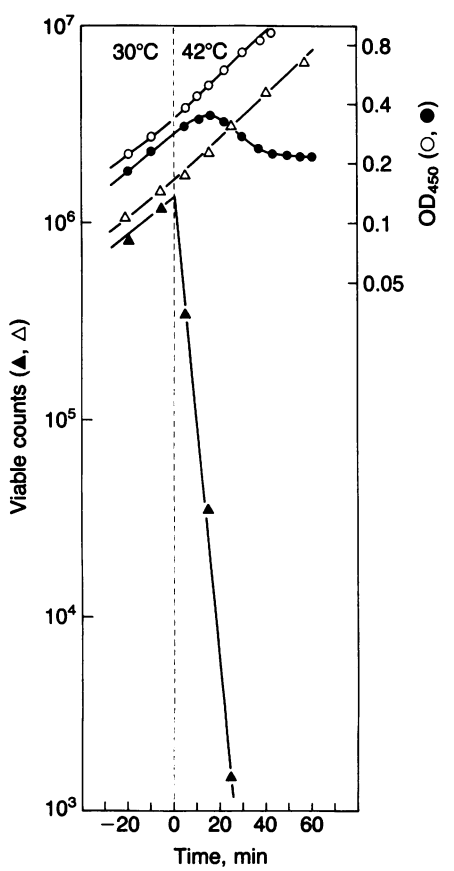

FIG. 4. Host cell killing after λ P_R-induced activation of the *hok* gene. Strain JC411 containing either pKG634 (closed symbols) or pKG171 (open symbols) was grown exponentially in A + B minimal medium supplemented with Casamino acids at 30°C. At time zero, the temperature was shifted to 42°C, and growth of the cultures was followed as OD_{450} and viable counts on selective medium (LB plates containing ampicillin at 50 μg/ml).

A second *parB*$^+$ plasmid (pPR633) was subsequently transformed into strain JC411 (pF634) and the induction experiment was repeated with this double plasmid strain. As seen in Fig. 5, the *parB*$^+$ region present in *trans* fully suppresses the induction of the *hok* gene. Thus, the *parB*$^+$ region must encode a *s*uppressor *o*f host *k*illing (the *sok* gene). Addition of the *parB*$^+$ region in *trans* also suppresses the effects on the host cells of the pSC101 *rep*(ts) *parB*$^+$ plasmid (pPR122) after a transfer to the restrictive temperature (not shown).

The position of the *sok* gene was determined by insertion of the λ cI_{857}–P_R fragment into a plasmid containing the left part of the *parB*$^+$ region (pPR171, Table 1), resulting in pKG171 (see Fig. 3). At 30°C, pKG171 does not express a Sok$^+$ phenotype, whereas induction of the P_R promoter at 42°C activates the *sok* gene of the plasmid, resulting in a Sok$^+$ phenotype (essentially shown as described above). This result indicates that the suppressor of *hok*-mediated host cell killing is encoded by the left-hand part of the *parB*$^+$ region, with transcription being in the opposite direction relative to that of the *hok* gene.

A more detailed description of the mapping of the *hok* and *sok* genes will be presented elsewhere (unpublished data).

DISCUSSION

The major finding presented here is the identification and partial characterization of a plasmid-encoded host cell killing activity that seems to be connected with stabilizing the inheritance of plasmid R1. In the light of the discoveries concerning stability functions of plasmid F, which revealed a coupling (*ccd*$^+$) between plasmid maintenance and cell division (3), we found it highly important to assess this possibility also for the R1 *par* genes. The observation that the presence of *parB*$^+$ on a plasmid had an influence on cell growth under conditions in which the copy number approached one per cell showed resemblance to the findings of Ogura and Hiraga

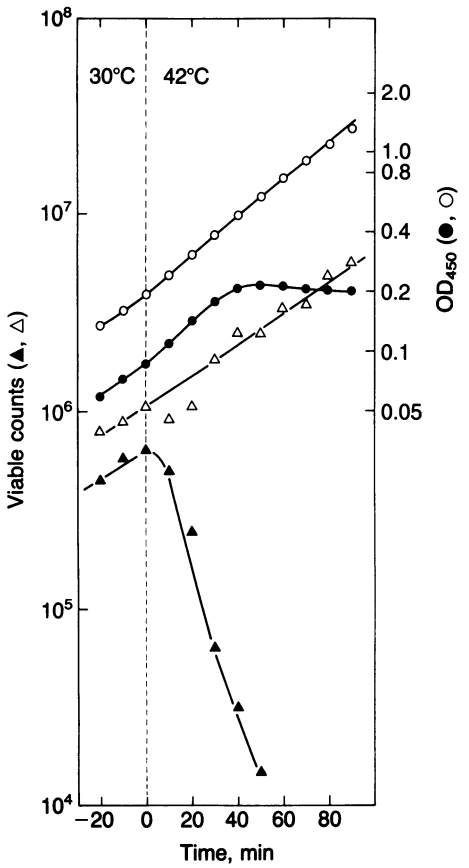

FIG. 5. Suppression of host cell killing. Strain JC411 containing either pF634 alone (closed symbols) or pF634 plus pPR633 (open symbols) was grown exponentially in A + B minimal medium supplemented with Casamino acids at 30°C. At time zero, the temperature was shifted to 42°C, and growth of the cultures was followed by measuring the optical density (OD_{450}) and viable counts on selective medium (LB plates containing kanamycin at 100 μg/ml).

concerning the *ccd* locus of F. Yet, for a number of reasons we do not believe that *parB* is a *ccd* analogue. (*i*) The presence of a $parB^+$ plasmid does not induce filamentation of cells in which the plasmid copy number approaches one per cell. (*ii*) The consequence of $parB^+$ activation is an irreversible killing of the host cell, which seems to be connected with the dramatic change in cell morphology (ghost cell formation). (*iii*) Shortly after the time when the copy number of a $parB^+$ plasmid has reached one per cell, the growth rate is reduced (see Fig. 1).

These observations do not match either the actual findings of Ogura and Hiraga or the predicted phenotype of *ccd*-like genes. We therefore suggest that $parB^+$ from plasmid R1 is responsible for a unique mechanism of "plasmid stabilization" involving rapid killing of the host cell. In order for such a mechanism to be most efficient, killing should only occur in cells that accidently have lost the $parB^+$ plasmid. The fact that dead ghost cells contain no plasmid DNA and that the number of plasmid-carrying cells stays constant in the absence of plasmid replication agrees with this expectation. Only under conditions in which the killing function is artifically overexpressed from a strong external promoter is killing observed in plasmid-containing cells. The term plasmid stabilization may seem inappropriate with respect to the proposed mechanism. We use this term to express that in a population of cells the occurrence of plasmid-free cells is greatly reduced or prevented by the $parB^+$ locus.

An important aspect of the mechanism by which *parB* exerts its effect is related to the question whether cell killing occurs before or after plasmid loss. We have presented data showing that, in the absence of plasmid replication, all cells that still have a copy of the plasmid survive. In contrast, the cells that die seem to harbor no plasmid DNA, which is an observation that does not indicate cell lysis since no β-galactosidase was lost from the cytoplasm to the growth medium (not shown). All evidence is therefore in favor of the proposal that if division of a $parB^+$-carrying cell results in one daughter cell still harboring the plasmid and another being plasmid-free, then only the latter is killed.

The actual occurrence of viable plasmid-free cells seen in Fig. 1*A* is most easily explained as "escape" from the killing. This low-level leakiness agrees with the greatly increased, but not complete, stability exerted by $parB^+$ on mini-R1 plasmids (7).

This picture of the *hok*-mediated killing is highly significant for understanding the *sok*-mediated immunity. If induction of killing activity only happens in the absence of the *hok* gene (the plasmid-free cell), then the *sok* product cannot act at the level of *hok* transcription, since any such control should also result in killing of the plasmid-carrying cells.

We therefore suggest as a working hypothesis that the *sok* product suppresses the *hok*-mediated killing by a mechanism that acts posttranscriptionally.

As a consequence of a plasmid stability mechanism involving postsegregational killing of plasmid-free cells, the $parB^+$ locus should stabilize any unstably inherited plasmid, no matter what causes the loss of the plasmid. This agrees with the finding that a large number of different plasmids, high copy number and low copy number, have been greatly stabilized by insertion of $parB^+$ fragments. Among those tested are p15, pBR322, RSF1010, *oriC* mini-chromosomes, mini-F—all of which for some reason had become unstable after genetic manipulation (unpublished data).

In conclusion, we claim that an important element in the maintenance functions responsible for the very stable inheritance of plasmid R1 is encoded by the $parB^+$ locus, from which a regulated host cell killing function is expressed in such a way that cells losing a $parB^+$ plasmid are killed. It would not be surprising if other plasmid systems encode similar host killing functions. Determination of the mechanism of cell killing awaits further investigation.

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